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Introduction

HER2 overexpression is a poor prognostic indicator in breast cancer. HER2 amplification is associated with early tumor dissemination, rapid tumor progression, and increased invasiveness, implying that HER2 has a significant role in the metastatic phenotype. We have demonstrated that two key steps in the metastatic mechanism, angioinvasion and transendothelial migration, are augmented by HER2 expression, and we have linked Angiopoietin-2, a vascular destabilizing protein, to expression of HER2. The **objective** of this research is to determine if the metastatic advantage of HER2 expressing cancer cells is imparted by Angiopoietin-2 production, and further to determine if overexpression of HER2 is linked to Angiopoietin-2 expression. The **scope** of this research begins with two assays to test 1) angioinvasion and 2) endothelial cell retraction, a key step in tumor-cell transendothelial migration. Using several strategies, the research protocol tests tumor cell production of Angiopoietin-2 or blockade of Angiopoietin-2 to determine if Angiopoietin-2 modulates the metastatic steps in question. Further, breast cancer specimens are tested for concurrent expression of HER2 and Angiopoietin-2, and also correlated with stage and grade of the tumor. In addition, concurrent expression of related receptors (Epidermal Growth Factor receptor, HER3, and HER4) are also tested for correlation of Angiopoietin-2 expression.

Body

Specific aim #1 calls for testing the effect of manipulating Angiopoietin-2 production in MCF-7 breast cancer cells on in vitro models of key metastatic steps. The first series of experiments tested tumor cell induced endothelial cell retraction, a key step in tumor cell transendothelial migration. Angiopoietin-2 expression was manipulated in these cells using 3 techniques: 1) Stimulating or blocking HER2 signaling using Herceptin® (a monoclonal antibody that binds to HER2 and prevents dimerization and signaling) or Heregulin β1 (a ligand for HER3 and HER4, which upon binding induces hetero-dimerization with HER2 and subsequent signaling;) 2) Direct application of Angiopoietin-2 into the assay or sequestration of tumor produced Angiopoietin-2 in the assay by treatment with soluble Tie-2/Fc receptor fusion protein; and 3) Angiopoietin-2 cDNA antisense transfection to decrease tumor cell production of Angiopoietin-2.

Experiment Series #1. Tumor cell induced endothelial cell retraction. These experiments tested whether Her2 signaling in MCF-7 breast cancer cells induced endothelial cell retraction during co-culture. Further, these experiments tested whether Ang-2 production by breast cancer cells is a key factor in the mechanism of EC retraction. MCF-7 cells or transfected MCF-7 cells that overexpress HER2 (HER cells) were used as the breast cancer model. Intact, 5-day-old monolayers of human iliac vein endothelial (HIVE) cells or human dermal microvessel endothelial cells (HMEC) were used as the endothelial cell model. We used Heregulin β1 to stimulate Her2 signaling or Herceptin® to block Her2 signaling.

YEAR 1

We showed that HER cells induce a greater degree of endothelial cell retraction, determined by A) number of tumor cells associated with endothelial cell retraction events, and B) the percent of subendothelial matrix that is exposed by retracting endothelial cells(1). We also showed that HER2 signaling induces endothelial cell retraction(1). MCF-7 or HER cells were pretreated with Herceptin® or Heregulin β1 to block or stimulate HER2 signaling. The MCF-7 cells were then cocultured onto the intact HIVE monolayers. Heregulin β1 treatment greatly increased the amount of endothelial retraction induced by coculture with MCF-7 cells. Conversely, endothelial cell retraction was effectively arrested by tumor cell treatment with Herceptin to block HER2 signaling (1).

We also showed that HER cells produce more Angiopoietin-2 than MCF-7 parental cells (2). These results implicated Ang-2, a vascular destabilizing protein involved in angiogenesis, in the mechanism of breast cancer cell-induced endothelial cell retraction. To test this hypothesis, we initially tested a semi-purified preparation of Angiopoietin-2 and directly applied the protein in escalating doses to the intact endothelial monolayers (HIVE) in the retraction assay. Further, MCF-7 cells were pretreated with soluble Tie2/Fc receptor fusion protein (sTie2/Fc) to bind and sequester tumor cell released Angiopoietin-2, in order to prevent Angiopoietin-2 interaction with the Tie-2 receptors on the endothelium. These cells were then cocultured onto the HIVE monolayers in the retraction assay. In these preliminary experiments, low doses of Angiopoietin-2 failed to induce significant retraction, but at 200 ng/ml, Angiopoietin-2 induced an extensive EC retraction, on scale with HER cells ($p < 0.01$ vs MCF-7) (3). Increasing doses of sTie2/Fc to sequester Angiopoietin-2 significantly altered the ability of MCF-7 cells to induce endothelial cell retraction at a dose of 200 ng/ml sTie2/Fc ($p < 0.05$ vs MCF-7) (3). These experiments suggest that Angiopoietin-2 is likely a factor in tumor cell induced endothelial cell retraction.

YEAR 2

To complete this experimental series, we proposed to test MCF-7 cells depleted of Ang-2 expression by antisense Ang-2 transfection in the EC retraction model. If Ang-2 depleted cells fail to induce EC retraction, the findings will strongly suggest a key role of Ang-2 in this mechanism. We created 3 clones with stable transfection of antisense Ang-2. To test for Ang-2 depletion, however, we needed to generate an appropriate anti-Ang-2 monoclonal antibody for the application using SELDI (Surface Enhanced Laser Desorption/Ionization) technology. Because the amount of Ang-2 protein produced by these cells is low, definitive depletion could not be accurately determined by standard Western blot, and we planned to screen by SELDI. We raised a functional anti-Ang-2 antibody. Recent improvements in Western blot detection proved sufficient to demonstrate lack of production of Ang-2 protein in these clones, and the SELDI tests were not necessary (figure 1).

YEAR 3

We completed the experiments studying the direct application of Ang-2 protein on EC monolayers. Another modification had to be introduced into these studies. We were no longer able to obtain primary cultures of HIVE cells. These cells were cultured from iliac veins harvested at the time of tissue procurement for liver transplantation, which were not used for that procedure. Commercially available HIVE cells (from Wistar via ATCC) were sporadic and would not form stable monolayers (likely due to overpassage). We transitioned to HMEC cells, a stable cell line of human dermal microvessel EC. After developing the appropriate cell conditions to replicate our other EC work, we tested the model with MCF-7 cells. MCF-7 cells were able to induce EC retraction in this model as well ($p < 0.05$ vs control). A commercially available, purified preparation of Ang-2 protein was then tested in the EC retraction assay. Direct application of Ang-2 protein to EC monolayers resulted in a dose dependent increase in EC retraction (measured by the area of matrix exposed by retracting EC; $p < 0.001$ at 50 ng/ml vs BSA control; $p < 0.05$ at 5 ng/ml) (figure 2). Because VEGF is known to increase vascular permeability, and also appears to be upregulated by Her2 signaling, we also tested VEGF in the EC retraction assay. VEGF alone did not induce EC retraction (figure 2). The addition of VEGF to Ang-2 in the retraction assay significantly abrogated Ang-2 induced retraction ($p < 0.01$ Ang2 50 ng/ml vs Ang2 50 ng/ml + VEGF 50 ng/ml). This result is consistent with published data suggesting that Ang2 alone promotes vascular destabilization, while the presence of VEGF with Ang2 facilitates angiogenesis (4).

YEAR 4

We have obtained an additional source of HIVE cell primary cultures, and confirmed the effect of Ang2 on EC retraction. Using HIVE monolayers, direct application of Ang2 induces EC retraction in a dose-dependent manner. This effect is evident at low-serum concentrations (2% FBS) but severely reduced at 15% FBS where there is no significant difference. We postulate that increasing levels of VEGF and Ang1 present in FBS modulate the effect in high serum. This postulate will be further tested using stripped serum and affinity purified serum using anti-VEGF and anti-Ang1 antibodies. These results were submitted in abstract to the American Association of Cancer Research annual scientific meeting.

To complete this experimental series, we are currently testing three clones of the Ang2 antisense transfected MCF-7 (MCF-7$^{-Ang2as}$) cells in the retraction assay. We hypothesize that these cells will lack the ability to induce EC retraction. Although the preliminary data is suggestive, we do not have enough experiments completed to perform the statistical analysis to support the hypothesis at this time. We anticipate completing these experiments by July, 2005. Taken together, the data generated from this experimental series will then strongly suggest that Ang2 production in MCF-7 cells, influenced by Her2 signaling, induces EC retraction. This conclusion would suggest that Ang2 action might be a therapeutic target to reduce the metastatic phenotype in some breast cancers. In support of this hypothesis, recently published research demonstrates

that Ang2 sequestration eliminates angiogenic induction in corneal angiogenesis model, and promotes tumor regression in a xenograft model (5).

Experiment series #2. The next series of experiments evaluated Her2 signaling and the role of tumor produced Angiopoietin-2 in the mechanism of angioinvasion through microvessel dismantling. Angioinvasion was studied using a 3 dimensional in vitro microvessel-dismantling assay of isolated rat microvessels embedded in collagen I gel (5).

YEAR 1

We showed that microvessels dismantle upon exposure to MCF-7 cells or HER cells. After coculture with these cells, embedded microvessels demonstrate areas of discontinuity, with architectural dismantling. We compared MCF-7 cells with HER cells, which express significantly more Angiopoietin-2 (5). HER cells induce a significantly more rapid and more extensive effect on microvessel dismantling ($p < 0.05$ vs MCF-7). To further implicate HER2 signaling as a mechanism for this metastatic step, we pretreated MCF-7 cells with Herceptin® or Heregulin β1 to block or induce HER2 signaling. Blockade or stimulation of HER2 signaling dose dependently limits or enhances tumor cell induced microvessel dismantling ($p < 0.01$). We further demonstrated that other HER2 expressing breast cancer cell lines can induce microvessel dismantling, and in at least one additional line the effect is blocked by Herceptin® blockade of HER2 signaling (5). We also tested the direct application of the semi-purified preparation of Angiopoietin-2 protein to induce microvessel dismantling. Further, we used sTie2/Fc to sequester tumor produced Angiopoietin-2. Microvessels in the dismantling assay were exposed to Angiopoietin-2 protein in increasing doses up to 200 ng/ml. No significant induction of microvessel dismantling was identified. We also treated the MCF-7 cells with sTie2/Fc to sequester Angiopoietin-2, and exposed the microvessels to these pretreated cells. sTie2/Fc dose-dependently-inhibited induced dismantling, reaching significance at 200 ng/ml ($p < 0.01$) although this effect was not dramatic (5).

YEAR 2

In year 2, we created 3 clones with stable transfection of antisense Ang-2 to limit protein production in the MCF-7 cells as described above. These cells will be used to confirm the results of the sTie2 sequestration experiments. We also raised a monoclonal antibody against Ang2 to test for loss of protein production in these cells.

YEAR 3

We tested the stable MCF-7^{Ang2as} cells for protein production and found no detectable protein by Western blot with enhanced detection (figure 1).

YEAR 4

We had proposed to test the direct application of Ang2 to the microvessels in culture in the microvessel-dismantling assay. These experiments are planned to be

completed by July 1, 2005 but no additional experiments have been completed in year 4. Although the preliminary experiments testing the partially purified Ang2 protein did not appear to impact the microvessels, we will confirm this result with the commercially available Ang2 protein. Further, we will compare the MCF-7^{Ang2as} and parental MCF-7 cells in the microvessel-dismantling assay. These experiments will complete this series. Based on our data obtain thus far, Ang2 has a role in Her2-signaling induced microvessels dismantling (as a model of angioinvasion), but it may be adjunctive. It appears that although Ang-2 may be key to EC retraction, the mechanism of Her2-induced microvessel dismantling may involve other Her2-signaling upregulated factors.

Experiment series #3. These experiments were not described in the Statement of Work, but were designed to further elucidate the mechanism of HER2 signaling and Angiopoietin-2 induction of endothelial cell retraction. We postulated that endothelial cells retract after the binding of Angiopoietin-2 to the Tie2 receptor on the endothelial cell because binding induces the dissociation of the catenin proteins from vascular endothelial (VE) cadherin. These proteins are key structural proteins of the adherens junctions of the endothelium. Under appropriate stimulation, a sequential dissociation of γ, β, and then α catenin from VE cadherin breaks the adherens junction link to the cytoskeleton, resulting in retraction and rounding of the endothelial cell.

YEAR 1

In these experiments, we tested intact human endothelial cell monolayers for dissociation of the catenins from VE cadherin after exposure to MCF-7, and further tested the MCF-7 cells after treatment with Herceptin and Heregulin β1 to manipulate HER2 signaling in these cells. Using immunoprecipitation of VE cadherin after exposure of the monolayer to tumor cells, we determined the quantity of the catenins, which remained associated with VE cadherin by Western blot analysis. Figure shows the quantity of the catenins linked to VE cadherin over time of exposure to MCF-7 cells. Western blots were digitized and the densitometric intensity was determined and compared to control. The data is reported as percent of control (untreated) monolayers. A time dependent loss of associated catenins is clearly demonstrated, with greater than 90% loss of γ catenin seen at 24 hrs ($p < 0.01$). Further, HER2 signaling regulation using Herceptin® and Heregulin β1 significantly altered the dissociation curve (figure 4). A 50% reduction in γ catenin dissociation was seen at 24 hrs after treatment with Herceptin®. Heregulin β1 significantly augmented MCF-7 induced γ catenin dissociation ($p < 0.05$), achieving equivalence with the result of HER cell induction of catenin dissociation. (Recall that HER2 signaling modulations alters the tumor cell production of Angiopoietin-2). These results imply that the mechanism of HER2 signaling induced endothelial cell retraction likely includes dissociation of the adherens junction proteins, with loss of continuity with the cytoskeleton.

Additional experiments tested Angiopoietin-2 sequestration with sTie2/FC from MCF-7 cells in coculture with endothelial monolayers. After pretreatment with increasing doses of Tie2/Fc, MCF-7 cells were cocultured with endothelial monolayers. After

immunoprecipitation with anti-VE cadherin antibody, Western blot analysis of the immunoprecipitate for γ catenin was performed. Figure 5 shows that sequestration of Angiopoietin-2 with sTie2/Fc significantly reduced the γ catenin dissociation induced by MCF-7 cells back to 50% of control ($p < 0.05$ vs MCF-7), similar to Herceptin® treatment. This experiment further implicates tumor cell produced Angiopoietin-2 as part of the mechanism of the HER2 signaling induced, metastatic phenotype.

YEAR 4

To complete this series of experiments, we tested the direct application of the commercially available Ang2 protein to EC monolayers to induce catenin dissociation. These final experiments are nearly completed, and we are currently evaluating the last experimental results. The preliminary review suggests that Ang2 protein induces EC retraction by dissociation of VE-cadherin and the catenins forming the adherns junction. This effect is dose dependent. With treatment of 200 ng/ml of Ang-2, significant dissociation of beta- and gamma- catenin from VE cadherin was seen (beta: 64.7% of control, $p < 0.01$; gamma: 78.2% of control, $p < 0.05$). These results will be included in a resubmission of this manuscript (3).

Specific Aim #2 calls for the determination of concurrent expression of HER2 and Angiopoietin-2 in breast cancer specimens with the aim to determine if HER2 expression is linked to Angiopoietin-2 expression in breast cancer. The method uses laser capture microdissection as described in the proposal protocols. Because HER2 is the signaling subunit of heterodimers with other types I growth factor receptors (EGFR, HER3, and HER4) we will also determine relative levels of these receptors in the breast specimens.

YEAR1

We collected over 50 cancer specimens, and have tested 11 cancers. We also tested three normal breast specimens along with placenta as a positive control. Of 11 breast cancers, 5 over-express HER2. Seven cancers express Angiopoietin-2, including all of the Her2 overexpressing cancers. This pilot data supports the HER2 link to Angiopoietin-2 production, and was published (2).

YEAR 2

Recent publications indicate that Her2 signaling (stimulated by Heregulin β1) upregulate expression of VEGF (6,7). Because Ang-2 is also a key factor in angiogenesis, particularly in the presence of VEGF, we expanded the scope of the research to include additional angiogenic factors. Figure 6 shows gene expression in several breast cancer cell lines, with relative expression of several key angiogenic factors. MCF-7 (2 separate strains, one estrogen dependent "MCF-7b", and one independent "MCF-7") MDA-MB-175, and SkBR3 cells all express Ang-2, while MDA-MB-231 cells do not. The –231 cells also do not express significant quantities of VEGF. The –231 cells also do not express Her3 or Her4, which are key hetero-dimers for Her2 signaling. We had prior published that –231 cells did not express Ang-2 although they

expressed Her2 (2). It appears plausible that these cells do not express Ang-2 because of lack of expression of Her3 or Her4. (Additionally, published reports indicate Her2/Her3 dimers may be responsible for VEGF upregulation in breast cancer cells.) (6,7) Also of interest, the –175 cells express Her2 and Her3, but not significant quantities of Her4, yet express VEGF and Ang-2. Further, this data indicates that the MCF-7 cells have limited expession of Ang-1, a vascular stabilizing protein that competitively binds with Ang-2 to the Tie2 receptor. We postulate that the lack of Ang-1 expression in MCF-7 cells contributes to the phenotypic expression of induced EC retraction seen in our model.

Because the data suggests an angiogenic response in these cells from Her2 signaling, we further expanded the scope of this project to perform microarray analysis for angiogenic factors in these cell lines (figures 7,8). We tested the MCF-7 cells for angiogenic factor gene expression using a GEArray chip containing 96 angiogenic genes. The microarrays were also run on RNA extracted from MCF-7 cells after treatment with Heregulin β1 to stimulate Her2 signaling, and Herceptin® to block Her2 signaling. Additionally, stimulation with Heparin Binding-Epidermal Growth Factor (HB-EGF, which induces EGFR homo-dimerization and EGFR-Her4 hetero-dimerization with possible alternate signaling) was also performed. Preliminary results indicate significant upregulation of several angiogenic factors after Heregulin β1 stimulation (including FGF2, neuropilin 1, and TIMP1). Stimulation of MCF-7 cells with HB-EGF also upregulated angiogenic factors including FGF1, FGF2, neuropilin 1, and VEGFB. Herceptin® treatment demonstrated down regulation of multiple factors including Ang-2, PDGF-B, MMP2 and MMP9, IL-8, and integrin β3. Microarray analysis for angiogenic factors is planned for several human cancers to identify correlation with the cell lines. Although this expanded scope cannot be completed in the time duration of this Her2 signaling project, the preliminary data may support justification for additional study of the mechanism of the Her2-signaling induced metastatic phenotype.

YEAR 3

Forty breast cancer specimens were collected for this study in prior years (in addition to the 11 cancers tested in the pilot experiments). These forty cancers were prepared for Laser Capture Microdissection (LCM) in year 3. Of these forty cancers, 17 were unevaluable. The primary reason was lack of sufficient cancer cells within the specimen. In 23 specimens, LCM isolated breast cancer cells (eliminating vascular elements), RNA was isolated and RT-PCR performed for Ang-2, VEGF, and actin. Additional tissue was used to isolate RNA for RT-PCR of the HER family receptors. Thirteen tissues were ER+, while 10 tissues were Her2+. Ang2 mRNA was detected in all specimens. This result was not anticipated in the original proposal, but a recent publication (8) demonstrated that Ang2 appears to be linked to estrogen binding to ER in breast cancer cells lines. With this new data, we chose to do an in study regression analysis of the data, despite not yet completing all the proposed tissues to reach appropriate power, in order to determine if the study should continue. Figure 9 shows the regression analysis of Ang2 to Her2 expression in Her2+ cancers ($R = 0.59$, $p < 0.07$). Although this correlation is not significant at the < 0.05 level, the study is underpowered to accept a negative conclusion. We should achieve sufficient power with the remaining

tissues we have proposed to study. If the slope remains similar, we anticipate statistical significance. An additional 15 tissues will be tested in the extension year. No correlation was seen with Her2 expression in the ER+ tumors. (Note: All breast tissues express low levels of the HER family of receptors. Over-expression of Her2 in breast cancer imparts an aggressive phenotype, which we have listed in this study as "Her2 +"). The differences in the slopes of these two regression analyses are highly statistically significant. These preliminary data suggest that Ang2 expression is linked to ER function in ER+ tumors (based on published data), but regulated by Her2 signaling in Her2 overexpressing cancers.

YEAR 4

We have not yet completed the LCM experiments proposed (an additional 27 tissues) to achieve statistical power for data analysis. We are awaiting IRB approval for the study at Moffitt. The tissues are reserved in the tissue bank pending approval of the study. We will also quantitate the estrogen receptors on the ER+ tumors to test the hypothesis that ER receptor expression correlates with Ang2 expression in ER+ tumors. To complete this experimental series, we will perform immunohistochemistry to show protein expression (Ang2, Her2) in these tissues. Ultimately, we will evaluate the TNM staging of these tumors and compare to Ang2 expession. These experiments are scheduled to be completed by July 1, 2005.

Key Research Accomplishments

- Determined that HER2 signaling induces a metastatic phenotype in breast cancer involving endothelial cell retraction (as a key step in transendothelial migration) and microvessel dismantling (as a potential avenue of angioinvasion.)
- Determined that Angiopoietin-2 can induce endothelial cell retraction, and is likely a key factor in the mechanism of the HER2 signaling induced metastatic phenotype
- Determined that the mechanism of endothelial cell retraction involves dissociation of the catenin proteins from VE cadherin, and loss of adherens junctional linkage to the cytoskeleton
- Determined that Angiopoietin-2 is likely involved in the mechanism of HER2 signaling induced microvessel dismantling, but is not the only factor in this mechanism.
- Identified that Angiopoietin-2 expression appears to correlate with Her2 expression in Her2 + breast cancers, although alternate-signaling pathways (ER) may also influence Angiopoietin-2 production in ER+ cancer cells.
- Identified that concurrent expression of Her3 may be required for Angiopoietin-2 (and VEGF) expression in breast cancer cells.
- Identified that several angiogenic factors appear to be regulated by Her2 signaling in MCF-7 cancer cells.

Reportable Outcomes

1. Carter WB. HER2 signaling-induced microvessel dismantling. *Surgery* 2001;130:382-387.
2. Carter WB, Ward MD. HER2 signaling-induced microvessel dismantling. Abstract. Presented at the Society of University Surgeons, February 10, 2001, Chicago, IL.
3. Carter WB, Hoying JB, and Williams SK. HER2 overexpression enhances tumor cell transendothelial migration. Abstract. Presented at the Society of Surgical Oncology, March 12, 2001, Washington, D.C.
4. Carter WB, Hoying JB, Boswell C, Williams SK. Her2/neu overexpression induces endothelial cell retraction. *International Journal of Cancer.* 2000; 88:295-299.
5. Carter WB, Ward MD, Hoying JB. Mechanism of HER-2 induced endothelial cell retraction. *Annals of Surgical Oncology.* Accepted for publication, but author generated major revisions will require resubmission.
6. Carter WB, Small G, Ward MD. Mechanism of Her2-induced endothelial cell retraction. Poster abstract. Presented at the Era of Hope, September 25-28, 2002, Orlando, FL.
7. Carter WB. Angiopoietin-2 induces endothelial cell retraction by dissociation of adherens junction proteins. For presentation at the AACR Annual Meeting, Anaheim, CA April 17, 2005 (Poster # 1154, Tumor Biology 10).

Conclusions

The work to date has substantially increased the knowledge available about the mechanisms involved in the development of a metastatic phenotype associated with HER2 overexpression. We have shown that at least two metastatic mechanistic pathways are enhanced by HER2 signaling; 1) endothelial cell retraction and transendothelial migration, and 2) microvessel dismantling as a portal for angioinvasion. Further, these metastatic pathways appear to involve Angiopoietin-2, a vascular destabilizing protein. The work presented identifies that the Angiopoietin-2/Tie-2 receptor pathway is likely a key intermediary step in the metastatic phenotype, and a worthy therapeutic target. Further, the determination of Angiopoietin-2 expression in breast cancer may suggest an appropriate tumor marker indicating greater metastatic or angiogenic potential. The remaining experiments designed in this study are likely to offer additional insights into these mechanisms, and perhaps elucidate other potential clinical targets or markers.

Unfortunately, delays in the completion of this project have been encountered. Recall to support the Global War on Terrorism by the PI led to delays in completion. Further delays have been encountered in obtaining a sub-contract to complete the work at the H. Lee Moffitt Cancer Center in Tampa, FL, where the PI has relocated the lab in October, 2003. The PI has initiated the research at Moffitt (unfunded at this time) to complete the

project by July 1, 2005. Two additional manuscripts reporting the results described are in preparation, and will incorporate the additional data sought from the remaining experiments. A final report will be submitted August 1, 2005 to the US Army Research and Materiel Command.

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